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Bacterial Growth Rate and Host Factors as Determinants of Intracellular Bacterial Distributions in Systemic *Salmonella enterica* Infections▽

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Bacteria of the species *Salmonella enterica* cause a range of life-threatening diseases in humans and animals worldwide. The within-host quantitative, spatial, and temporal dynamics of *S. enterica* interactions are key to understanding how immunity acts on these infections and how bacteria evade immune surveillance. In this study, we test hypotheses generated from mathematical models of in vivo dynamics of *Salmonella* infections with experimental observation of bacteria at the single-cell level in infected mouse organs to improve our understanding of the dynamic interactions between host and bacterial mechanisms that determine net growth rates of *S. enterica* within the host. We show that both bacterial and host factors determine the numerical distributions of bacteria within host cells and thus the level of dispersiveness of the infection.

Bacteria of the species *Salmonella enterica* are a threat to public health, causing enteric systemic diseases (typhoid and paratyphoid fever), gastroenteritis and nontyphoidal septicemia in humans and similar diseases in many other animal species worldwide. The emergence of multidrug-resistant *Salmonella* strains and the insufficient efficacy of some of the currently available *Salmonella* vaccines highlight the urgent need for improved prevention strategies to combat salmonellosis in humans and animals.

S. enterica typhoidal infections have a truly dispersive nature. In mouse infections, the clonal and independent expansion of bacteria derived from the initial inoculum is paralleled by an increase in the number of infected cells and of the multicellular pathological lesions that form at foci of infection (11, 15, 16). Control of the growth and spread of the bacteria requires recruitment and activation of phagocytes at the foci of infection with an influx of bone marrow-derived cells into the tissues and the concerted action of key cytokine networks: tumor necrosis factor alpha, gamma interferon (IFN-γ), and interleukin-12 (IL-12), IL-18 and IL-15 (7–9). In addition, reactive oxygen intermediates and reactive nitrogen intermediates act in phagocytes to control the growth of ingested bacteria (10, 17).

A key feature of the spread of *S. enterica* in the tissues is the heterogeneous numerical distribution of intracellular bacteria with low bacterial numbers seen at any one time in the majority of infected phagocytes, while a minority of phagocytes can be heavily infected (16). The combination of intracellular bacterial distributional data with "branching process" mathematical models allowed the discrimination of a number of mechanistic hypotheses on the cause of this strikingly skewed distribution of bacteria among host cells (1). In particular, the analysis gave scant support to the hypothesis that highly infected cells were intrinsically more susceptible to infection. Rather, the model supported the idea that all phagocytes were equivalently susceptible and that the skewed distribution was generated by the epidemic spread of the bacteria through the host cell population. The model gave further discrimination at the level of specific characteristics of within-cell bacterial division and cell death, with most support offered for a model with a constant rate of stochastic cell lysis of infected phagocytes, independent of intracellular bacterial numbers, and density-dependent slowing in intracellular bacterial division rates (1).

The use of our mathematical models as analytical platforms which allow us to elicit information about unobserved processes from observed data (1, 3) can be extended to generate and test predictions on the mechanistic bases of microbial and host determinants of bacterial spread and infection dynamics. For example, our previous model (1) predicts faster-growing strains to have distributions of intracellular bacteria that are more skewed toward higher counts. The relationship between bacterial growth and distributional bias can be understood as a consequence of an increase in the risk of host-cell lysis for a given bacterial load in those infections in which the bacteria grow more slowly; under these conditions, the intracellular bacterial load takes longer to reach a given size, so the likelihood of lysis before reaching that size will be greater.

In this report, we test whether variations in net bacterial growth determined by microbial factors or host immune control mechanisms affect intracellular bacterial loads per host phagocyte. The data indicate that intracellular bacterial loads in vivo correlate with the intrinsic growth rate of the bacteria in a given host-pathogen combination and/or experimental condition.

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MATERIALS AND METHODS

Animals. C57BL/6 mice were purchased from Harlan Olac, Ltd. (Blackthorn, Bicester, United Kingdom). *gp91*$^{-/-}$ phox mice were bred in the Cambridge animal unit from breeding pairs generously provided by Jennie Blackwell (18). Mice homozygous for deletions in IFN-γ receptor (IFN-γR$^{-/-}$) were housed at Central Biological Services, Imperial College, London, United Kingdom, and were a kind gift from G. Dougan. Sex- and aged-matched mice between 9 and 14 weeks old were used for experiments. Experiments were covered by a Project License granted by the Home Office under the Animal (Scientific Procedures) Act 1986. This license was approved locally by the University of Cambridge Ethical Review Committee.

Bacteria. *S. enterica* serovar Typhimurium strain SL3261 is an *aroA*-attenuated derivative of *S.* Typhimurium SL1344 with an intravenous (i.v.) 50% lethal dose for BALB/c mice of ~$\log_{10}$ 7 CFU (8). *S.* Typhimurium C5 is a highly virulent strain with an i.v. 50% lethal dose of <10 CFU in BALB/c mice (6). For the "single/double" experiments, we used rifampin (rifampicin)-resistant SL3261 (O9) and nalidixic acid-resistant C5 (O4), generated by selection on antibiotic-containing media. Bacterial suspensions for injection were grown for 16 h as a stationary culture at 37°C in LB broth. Bacteria were diluted in phosphate-buffered saline (PBS) prior to injection into a lateral tail vein.

Enumeration of viable salmonellae in the tissue. Mice were killed by cervical dislocation, and the livers were aseptically removed, individually weighed, and divided in half. Half of each organ was homogenized (separately) in a Seward stomacher 80 Biomaster (Seward) in 10 ml of distilled water, and viable bacterial counts in the homogenate were assayed on pour plates of LB agar, supplemented with rifampin (100 μg/ml) or nalidixic acid (15 μg/ml) as appropriate.

Immunostaining for fluorescence microscopy. Half of each organ was fixed overnight in 4% paraformaldehyde diluted in PBS, washed for 1 h in two changes of PBS, and then immersed in 20% sucrose (in PBS) for 16 h at 4°C before being embedded in Optimal Cutting Temperature (OCT; Raymond A Lamb, Ltd., Eastbourne, United Kingdom) in Cryomoulds (Park Scientific, Northampton, United Kingdom). Samples were frozen by submerging in liquid nitrogen-cooled isopentane (2-methylbutane) and stored at −80°C. Thirty-micrometer sections were cut, blocked, and permeabilized for 10 min in a permeabilizing solution containing 10% normal goat serum diluted in PBS containing 0.02% saponin (Sigma, Poole, United Kingdom). For the experiments involving wild-type versus *gp91*$^{-/-}$ phox mice and wild-type versus IFN-γR$^{-/-}$ mice, sections were stained with 1:100 anti-CD18^{+} rat monoclonal antibody (clone WT.3; BD Bioscience) together with 1:100 Alexa Fluor 488-conjugated anti-lipopolysaccharide (anti-LPS) O4 monoclonal antibody (Custom Synthesis, Molecular Probes, OR), both diluted in permeabilizing solution, for 16 h at 4°C. Subsequently, sections were washed in PBS (two 30-min washes) and then incubated with 1:200 goat anti-rat Alexa Fluor 568-conjugated antibody (Invitrogen-Molecular Probes, Paisley, United Kingdom) diluted in PBS for 45 min. In the "single/double" infections, sections were stained with 1:100 anti-CD18^{+} rat monoclonal antibody (clone WT.3; BD Bioscience) together with 1:100 Alexa Fluor 488-conjugated anti-LPS O4 monoclonal antibody (Custom Synthesis, Molecular Probes, OR) and 1:200 anti-LPS O9 agglutinating serum, diluted in permeabilizing solution, for 16 h at 4°C. Subsequently, sections were washed in PBS (two 30-min washes) and then incubated with 1:200 goat anti-rabbit Alexa Fluor 568-conjugated antibody (Invitrogen-Molecular Probes, Paisley, United Kingdom) and 1:200 goat anti-rat–Alexa Fluor 647-conjugated antibody (Invitrogen-Molecular Probes, Paisley, United Kingdom). All sections were washed in PBS (three times for 15 min each) and mounted onto Vectabond-treated glass slides (Vector Laboratories, Ltd.) using Vectashield containing DAPI (4′,6-diamidino-2-phenylindole; Vector Laboratories, Ltd.). The analysis of tissue sections was by multicolor fluorescence microscopy using a Leica DM6000B fluorescent microscope running FW4000 acquisition software. Intracellular bacterial distributions were based on the counts obtained from 650 to 1,200 infected liver phagocytes from liver tissue obtained from five to seven mice.

Statistical analysis. Differences in mean $\log_{10}$ CFU counts were compared using a two-sample *t* test. To explore whether there were increasing or decreasing trends in the proportions of intracellular bacteria between two competing groups, we performed chi-square tests for trend. These are more powerful than conventional chi-square tests when there is an implicit ordering to the groups (in this case the number of intracellular bacteria), since it explicitly tests for linear trends in the data. We classified intracellular bacterial numbers into six groups: 1, 2, 3–5, 6–10, 11–15 or >15 bacteria/phagocyte. All analyses were carried out using the R statistical language (14).

RESULTS AND DISCUSSION

Ablation of host resistance factors affects *S. enterica* distribution: roles of phagocyte NADPH oxidase and IFN-γ. To determine the role of host immunological factors in shaping the intracellular numerical distribution of *S. enterica* cells in the murine liver, we infected mice deficient either in phagocyte NADPH oxidase or in the receptor for the phagocyte-activating cytokine IFN-γ.

During a systemic *S. enterica* infection, reactive oxygen intermediates generated via the phagocyte NADPH-oxidase (phox) are produced at the foci of infection and are highly bactericidal in the early stages of the infection, becoming bacteriostatic as the infection progresses (3, 10, 17). To reveal if the oxidative burst plays an important role in determining the bacterial distributions in host cells, we injected *gp91*$^{-/-}$ phox mice, functionally deficient in the oxidative burst, together with matched wild-type C57BL/6 controls with $\log_{10}$ 5.93 CFU of SL3261. We determined that at 5 days postinfection (p.i.), the mean bacterial load per liver was on average $\log_{10}$ 2.2 CFU greater ($P = 1.47 \times 10^{-9}$) in the *gp91*$^{-/-}$ phox mice than in the wild-type mice (*gp91*$^{-/-}$ phox mice, $\log_{10}$ 7.81 ± 0.19 CFU; wild-type mice, $\log_{10}$ 5.61 ± 0.07 CFU [mean ± standard deviation]). The distributions of the numbers of bacteria per cell were also different between wild-type and *gp91*$^{-/-}$ phox mice (Fig. 1A), with the *gp91*$^{-/-}$ phox phagocytes exhibiting higher bacterial loads on average. We can summarize the relationships depicted in Fig. 1 through the use of odds ratios (ORs). For example, for a single group (e.g., C57BL/6), the odds of having two bacteria per cell relative to one bacteria per cell are simply the number of cells containing two bacteria divided by the number containing a single bacterium. Therefore, the odds give a comparative measure of how likely the two events (two bacteria per cell versus one bacterium per cell) are within each group. If we have more than one group (i.e., C57BL/6 versus *gp91*$^{-/-}$ phox mice), we can use the ratio of the odds between each group to summarize the relative odds. Therefore, an OR of 1 means that the odds of having two bacteria per cell compared to one bacterium per cell are the same for both the C57BL/6 mice and the *gp91*$^{-/-}$ phox mice. An OR value of <1 would indicate that there is a larger odds of having more bacteria per cell in the knockout mice than in the wild-type mice, and an OR value of >1 indicates the opposite. All ORs in Table 1 are relative to the group containing a single bacterium. The top row in Table 1 shows the trend in higher intrabacterial numbers for the *gp91*$^{-/-}$ phox phagocytes ($P = 1.07 \times 10^{-5}$).

IFN-γ is produced by NK cells, T lymphocytes, and macrophages and is important in controlling early *Salmonella* growth in the host, principally via bacteriostatic mechanisms (4, 5, 12, 13). IFN-γR$^{-/-}$ mice, which lack the receptor for IFN-γ signaling, and C57BL/6 wild-type control mice were infected with $\log_{10}$ 5.81 CFU of SL3261. At 12 days p.i., the mean bacterial load per organ was on average $\log_{10}$ 3.76 CFU greater ($P = 9.56 \times 10^{-9}$) in the IFN-γR$^{-/-}$ mice than in the wild-type mice (IFN-γR$^{-/-}$ mice, $\log_{10}$ 9.17 ± 0.05 CFU; wild-type mice, $\log_{10}$ 5.41 ± 0.59 CFU [mean ± standard deviation]). The distributions for intracellular bacterial loads between the wild-type and IFN-γR$^{-/-}$ mice are shown in Fig. 1B. The trend in the data (middle row in Table 1) is that the IFN-γR$^{-/-}$ phago-

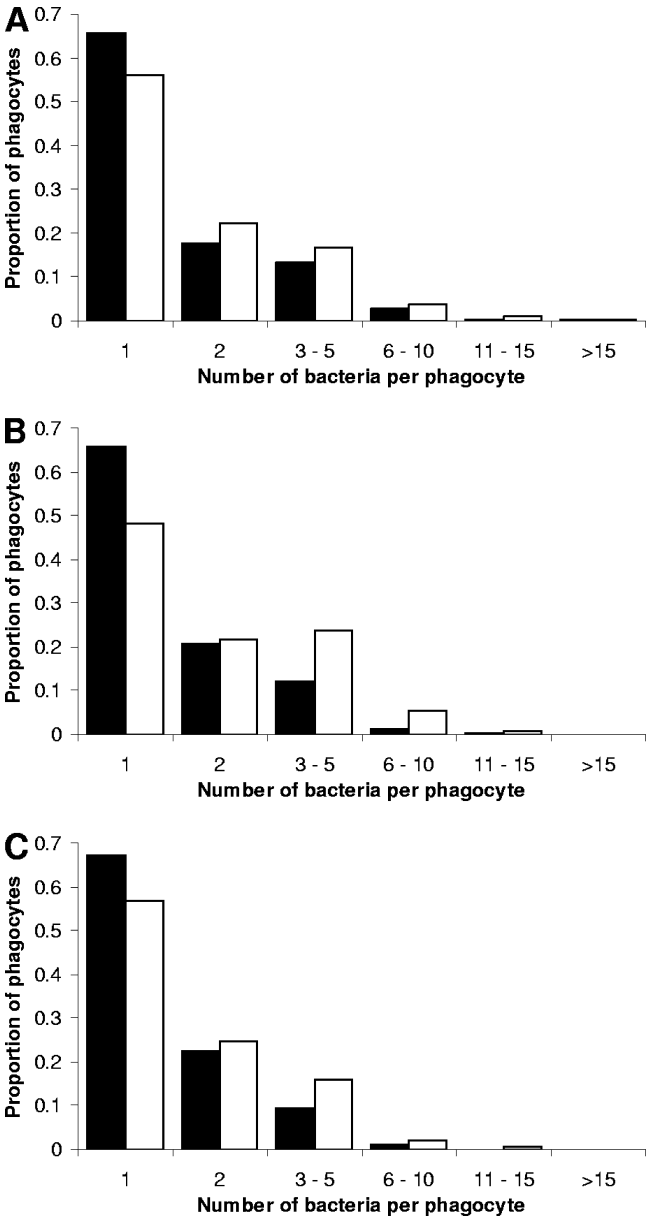

FIG. 1. Intracellular distribution of bacteria within infected phagocytes in the liver during infection of wild-type and knockout mice. (A) C57BL/6 control mice (black bars) and *gp91*$^{-/-}$ phox mice (white bars) at 5 days p.i.; (B) C57BL/6 control mice (black bars) and IFN-γR$^{-/-}$ mice (white bars) at 12 days p.i.; (C) single (black bars) and double (white bars) infection of C57BL/6 mice at 3 days p.i.

cytes exhibit higher intrabacterial loads than those from the wild-type mice ($P < 2.2 \times 10^{-16}$).

These results indicate that when bacterial growth is accelerated because of the lack of immune control mechanisms in the *gp91*$^{-/-}$ phox and IFN-γR$^{-/-}$ mice, the distribution of bacteria within host phagocytes is skewed toward higher numbers of bacteria per cell.

Exacerbation of the net bacterial growth of virulent *S. enterica* in host phagocytes results in higher intracellular densities. The experiments described above indicate that exacerbation of the growth of a slow-growing *S. enterica* strain (SL3261) due to impairment of the immune system results in increased intracellular numerical distributions. We also know from our previous work that virulent, fast-growing strains show a skew toward higher intracellular intensities than slow-growing attenuated strains (1). Here we wish to address the question of whether further acceleration of the already rapid growth of a virulent strain in innately susceptible mice would result in higher intracellular bacterial densities than those normally seen in virulent infections. To address this question, we used a system in which parenteral coadministration of an attenuated *S. enterica* vaccine strain (SL3261) at a high dose can increase the growth rate of a virulent strain (C5) in the tissues (2). This effect is dependent on the release of the immunosuppressive cytokine IL-10 by way of stimulation of Toll-like receptor 4 (TLR4) acting through signaling pathways involving both TRIF and MyD88 adaptor molecules (2).

Mice were injected with $\log_{10}$ 3.17 CFU of nalidixic acid-resistant C5 ("single" infection) or $\log_{10}$ 3.17 CFU of nalidixic acid-resistant C5 and $\log_{10}$ 6.08 CFU of rifampin-resistant SL3261 ("double" infection). We determined that at 3 days p.i., the mean load of C5 per organ in the double-infection mice was greater than that in the single-infection mice: $\log_{10}$ 8.1 CFU and 6.2 CFU, respectively. (At 3 days p.i., the mean load of SL3261 per organ in the double-infection mice was $\log_{10}$ 6.1 CFU.) The ORs and distributions are shown in Table 1, bottom row, and Fig. 1C, respectively. The plot shows a shift toward higher bacterial loads per cell for the double compared to the single infections, backed up by the chi-square test for linear trend ($P = 4.58 \times 10^{-6}$). These results suggest that the increased growth rate of the C5 strain in the double infection relates to increased numbers of bacteria per phagocyte.

The work presented in this paper shows that intracellular bacterial loads in vivo increase with the intrinsic growth rate of the bacteria in a given mouse strain. The distribution of intracellular density for a given *S. enterica* strain becomes skewed to higher numbers if the infection is performed in immunodeficient animals (e.g., *gp91*$^{-/-}$ phox and IFN-γR$^{-/-}$ mice), where

TABLE 1. Differences between groups in the proportion of total infected host CD18^{+} phagocytes containing various numbers of bacteria as tested by a chi-square test for trend

Group comparison	OR for no. of bacteria/phagocyte shown[a]:						*P* value
	1	2	3–5	6–10	11–15	>15	
C57BL/6 vs *gp91*$^{-/-}$ phox (day 5 p.i.)		0.68	0.69	0.59	0.297	0.68	1.07×10^{-5}
C57BL/6 vs IFN-γR$^{-/-}$ (day 12 p.i.)		0.69	0.37	0.19	0.14	0	$<2.2 \times 10^{-16}$
Single vs double infection (day 3 p.i.)		0.76	0.49	0.46	0	–	4.58×10^{-6}

[a] Each column shows the OR between the groups (i.e., control versus test) relative to one bacterium per cell. The chi-square test for trend assesses the existence of this trend in the ORs.

the bacteria increase in numbers at a higher rate. *S. enterica* strains with increased ability to grow in the tissues show distributional patterns skewed toward greater intensities of intracellular infection (i.e., higher numbers of bacteria per cell on average). These intracellular bacterial distributions are consistent with a model in which the burst rate of infected cells is independent of the number or net growth rate of the bacteria within the cells (1). Our findings indicate that subtle interactions between bacteria and the host immune system, in addition to the intrinsic net growth rate of a bacterial strain, determine the spread of *Salmonella* in the tissues.

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